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CITATION:

Tsuji, Masaki. Hydrophilization of Specimen Supports using Glow Discharge for Transmission Electron Microscopy. Bulletin of the Institute for Chemical Research, Kyoto University 1993, 70(5-6): 510-518

ISSUE DATE:

1993-02-26

URL:

http://hdl.handle.net/2433/77478

Bull. Inst. Chem. Res., Kyoto Univ., Vol. 70, No. 5-6, 1993

Note

Hydrophilization of Specimen Supports using Glow Discharge for Transmission Electron Microscopy

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Received December 11, 1992

KEY WORDS: Transmission electron microscopy/ Hydrophilization/ Ion bombardment/ Glow discharge

INTRODUCTION

In biological transmission electron microscopy, uniform spreading of the specimen suspended in water and/or of the aqueous solution of negative staining agent is very important to obtain sharp images on support films such as of evaporated carbon, of Formvar (polyvinyl formal) and of Collodion (nitrocellulose). Freshly made films are rather hydrophilic, but after leaving them for a long period even under vacuum, they have been hydrophobic. The hydrophobicity of the support film surface induces uneven spreading of the specimen and negative stain. In particular, uneven spreading of negative stain not only obstructs the fine details in the specimen, but also often breaks down even a thick support film. It is much troublesome to use freshly made support films all the time on specimen preparaion.

In the literature we find the methods of adding detergents such as Bacitracin[1,2] or other chemicals[2,3] as wetting agents either to aqueous suspension of the specimen or to an electron microscope grid where a support film has been deposited. Treatment of specimen suspensions or support films with various additives or wetting agents should, however, be avoided in high-resolution work, since they may add to the support noise and easily interfere with or obscure finer structural details.[4]

Another proposed method is irradiation of the specimen support surface with ultraviolet[5] or in a glow discharge (namely, ion bombardment)[4,6-9]. The glow discharge of an electron microscope grid coated with a support film, such as of evaporated carbon, of Formvar and of Collodion, prior to specimen deposition has a major advantage over chemical methods mentioned above, since it basically does not add anything to the support film.[4]

Here the preliminary results with a glow discharge apparatus are reported for the transmission electron microscopy of cellulose microfibrils.

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EXPERIMENTAL

As a test specimen, bacterial cellulose (synthesized by *Acetobacter Xylinum*) was used. Small pieces of cellulose membranes which were produced in culture medium[10] were washed several times with distilled water, and soaked and boiled for a few minutes in a 5 wt% aqueous solution of NaOH. After neutralization and washing with water, the sample was disintegrated and dispersed in distilled water by ultrasonification (Sonifer model 350) for about 6 minutes. The disintegrated sample was diluted with distilled water to obtain an appropriate concentration for the preparation of electron microscope grids.

A drop of the sample was placed on a 400-mesh grid coated with a carbon support film (10 ~ 20 nm in thickness): carbon was evaporated onto a freshly cleaved surface of mica and the resulting carbon film was deposited on the grid using a water surface. All carbon-coated grids used in this report had been prepared in advance and left for a long time on exposure to air. The grid was drained with filter paper and the specimen was stained by applying a drop of 2 wt% aqueous solution of uranyl sulfate[11]; about half a minute later the excess stain was removed by draining with filter paper and the specimen allowed to dry completely.

Just before deposition of the specimen on a carbon-coated grid, the grid was irradiated in a glow discharge using *Plasmaglo* optionally equipped to a vacuum coater (Edwards Model 306). Operating condition is shown in Table 1: only a mechanical vacuum pump was used. Vacuum condition was controlled by opening the High Vacuum Valve (main valve) by 1/4 turn and also carefully adjusting the Gas Inlet Valve (capillary valve) for small admission of air. A liquid N_2 trap was used to supress the counterflow of vapour of vacuum pump oil.

A Philips 400T transmission electron microscope was operated at 100 KV. Bright field images were recorded on Kodak electron microscope film 4489 at a magnification of 22,000 in principle.

RESULTS AND DISCUSSION

Figure 1 shows typical examples of uneven spreading of negative stain due to the hydrophobicity of support film surface. Carbon-coated grids were used without glow discharging. Figures 1-a and 1-b are the worst cases. A ribbon-like microfibril of cellulose (a bundle of finer microfibrils, i.e., 'elementary fibrils' or 'protofibrils' if they exist) is wrapped up in rather thick stain and it is difficult to recognize individual finer microfibrils in a ribbon. Under such a spreading condition, a support film itself is often broken down even if it is fairly thick. Figures 1-c and 1-d show better spreading than Figures 1-a and 1-b, but still in thick stain the ribbons of cellulose are wrapped. Somewhere aggregates of stain are distributed like islands (black spots on white background; typical examples are indicated by arrows and a letter A in Figures 1-c and 1-d). Moreover, there are many bubbles in the regions of rather thick stain (bright spots or disks on dark background; typical examples are shown by arrows and a letter B in Figures 1-c and 1-d). Details in a ribbon can be recognized slightly more clearly than in Figures 1-a and 1-b, but stain is still too thick and uneven for high-resolution observation.

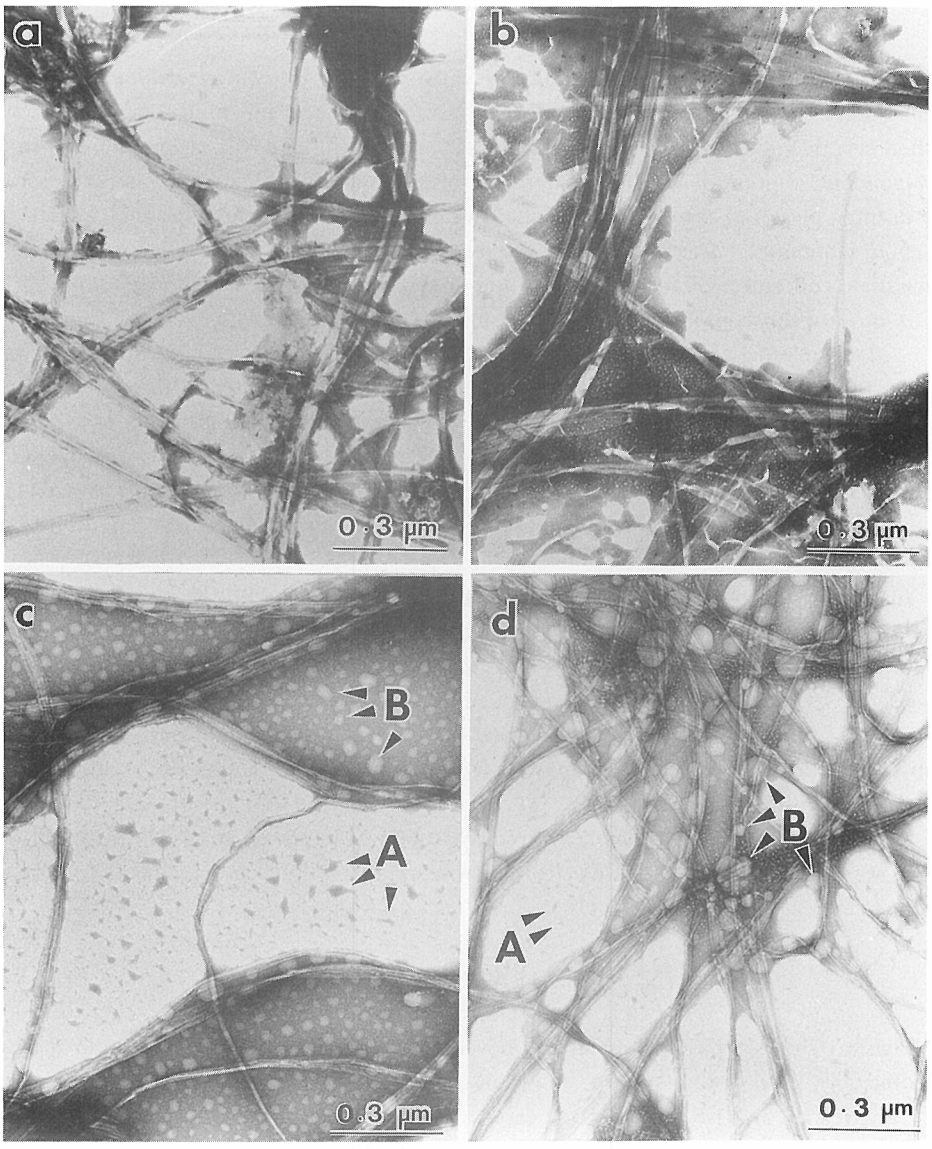

Figure 1. Typical examples of uneven spreading of a negative stain due to the hydrophibicity of specimen support films (without glow discharging).

Figures 2 and 3 show the results of a glow discharge (Figure 2 corresponds to 30 ~ 60 sec irradiation and Figure 3 to 120 sec irradiation under the condition shown in Table 1). As shown in Figure 2-a, regions of even and uneven spreading of stain sometimes coexist in the case of 30 ~ 60 sec irradiation of a glow discharge (for example, the regions of uneven spreading are indicated by arrows). In Figure 2-b, except the region of strongly aggregated

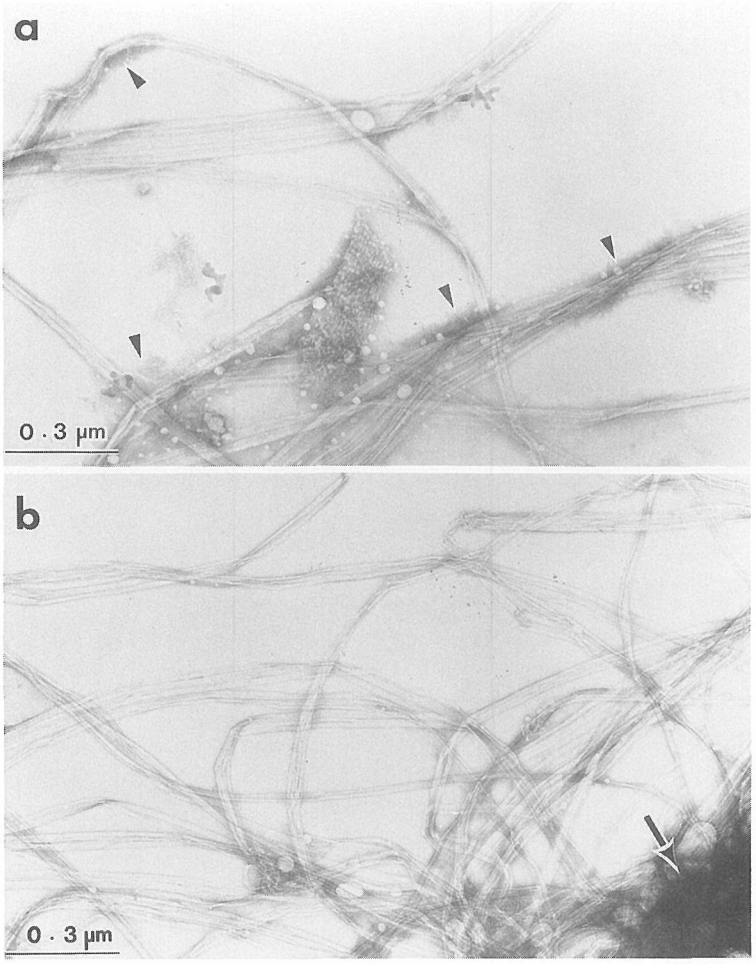

Figure 2. The results of 30 ~ 60 sec irradiation of specimen supports in a glow discharge.

Table 1 Operating Condition of a Glow Discharge *Plasmaglo* for Hydrophilic Treatment of Support Films in Transmission Electron Microscopy

Reading of a Pirani gauge of a vacuum coater (Edwards Model 306)	Electrode voltage (DC.KV)	Current (mA)
30 Pa (~ 0.2 Torr)	1 ~ 2	~ 40

ribbons of cellulose (indicated by the arrow), uniform spreading of stain can be recognized. If one wants to use a very thin support film (less than 5 nm in thickness) deposited on a

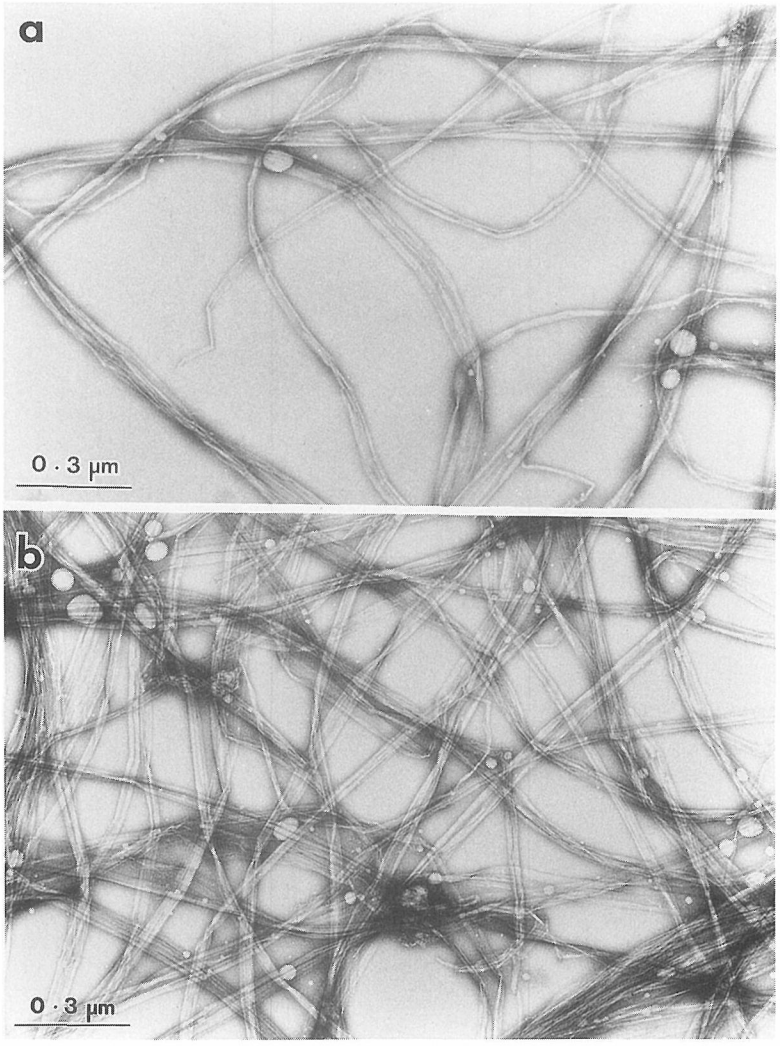

Figure 3. The results of 120 sec irradiation (optimal irradiation time) of specimen supports in a glow discharge.

gold-coated 'Triafol' microgrid, irradiation should be limited up to 60 sec because long irradiation in a glow discharge breaks down such a thin film.[9)]

Figure 3-a and 3-b are examples of 120 sec irradiation. Under the condition, spreading of stain is quite uniform on the whole grid. Fine details can be recognized in high contrast. For thick support films (more than 10 nm thick), 120 sec irradiation should be recommended. Longer irradiation beyond 120 sec did not show any differences.

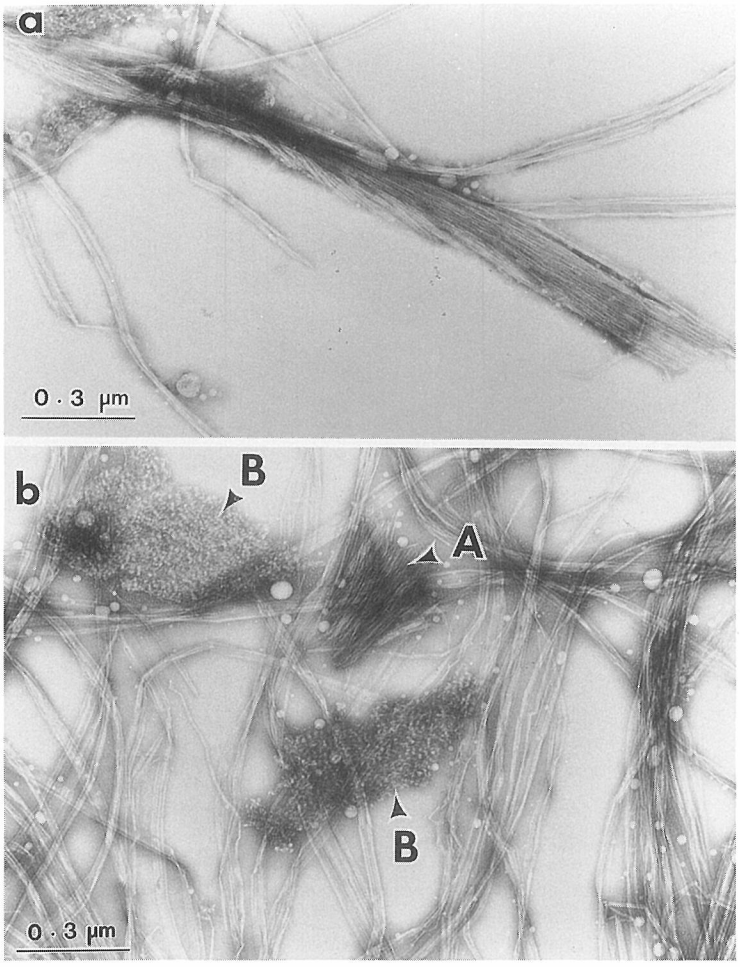

Figure 4. Examples using a gold-coated 'Triafol' microgrid covered with a very thin carbon film (abour 5 nm thick) which is irradiated for 60 sec in a glow discharge.

Figure 4 shows some examples using a microgrid coated with a very thin carbon film which was irradiated for 60 sec in a glow discharge. The carbon film was about 5 nm in thickness and was produced by indirect evaporation.[12] Figure 4-a shows a sheaf-like bundle of very fine microfibrils of cellulose. Individual fine microfibrils can be clearly recognized. Such a sheaf-like bundle of fine microfibrils also can be seen in Figure 4-b (it is indicated by a letter A in Figure 4-b), but it is very short (about 0.3 μm in length), probably due to ultrasonification. The objects indicated by a letter B in Figure 4-b seem to be wrecks of a 'mat', which had originally an intrinsic appearance like a rope curtain.[10]

Figure 5 shows an example of the effect of defocus on the apparent morphology of fine microfibrils of cellulose. Those images were recorded at a magnification of 46,000 using a very thin carbon film (about 5 nm thick) on a gold-coated microgrid: the film had been tre-

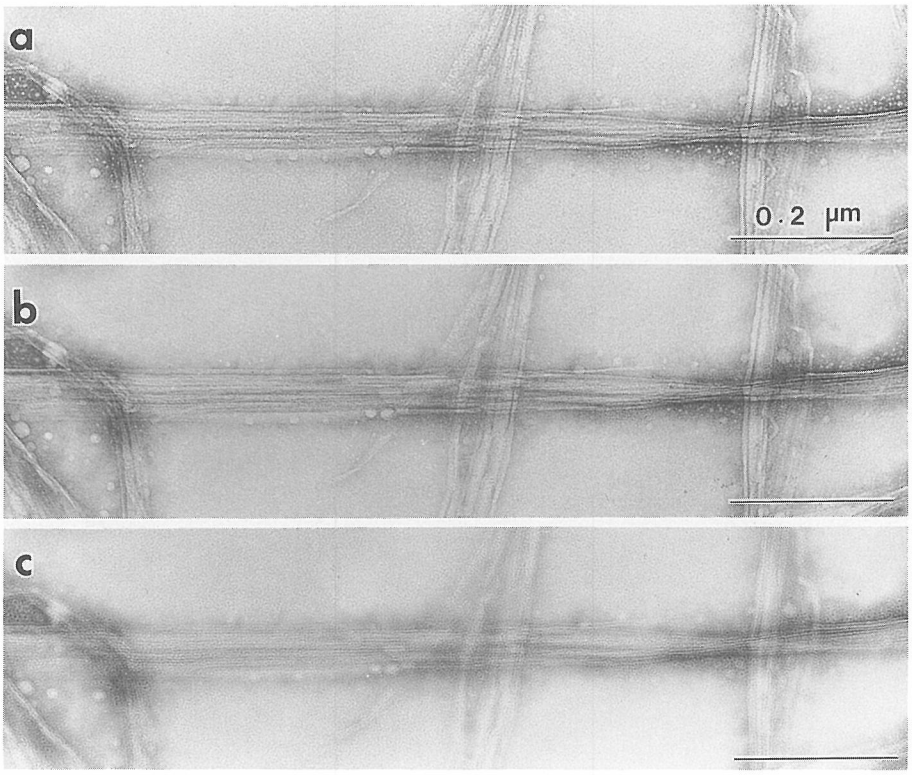

Figure 5. An example of defocus effect on the apparent morphology of a cellulose microfibril. Images were taken at a magnification of 46000 and at different levels of defocus.
a: about 1300 nm under-focus
b: almost in-focus
c: about 1500 nm over-focus.

ated for 60 sec in a glow discharge. Figure 5-a was taken at about 1300 nm under-focus (weak lens) and Figure 5-c at about 1500 nm over-focus. Individual fine microfibrils can be seen more clearly in Figures 5-a and 5-c than in Figure 5-b, which was taken at almost in-focus condition. Somewhere in Figures 5-a and 5-c, so-called beaded structure can also be recognized. The images of parallel arrays of fine microfibrils can be explained in terms of phase contrast, when a negative stain spreads out uniformly and thinly.[13] Though Huxley and Zubay reported that extreme hydrophilicity is not suitable because the negative staining solution then spreads out so thinly and evenly as to give low contrast around the particle,[3] it is thin and even spreading of a negative stain that must be desired for high-resolution observation. Thus a glow discharge should be recommended as a powerful method for the transmission electron microscopy of cellulose microfibrils.

Final thickness of a negative stain can be controlled by changing the concentration of the aqueous solution of stain. For uranyl sulfate, preliminary experiments indicated that the 2 wt% solution is the best for ordinary usage. In the case of high-resolution study of

very fine microfibrils of cellulose, however, somewhat more diluted solutions (1 ~ 1.5 wt%) seem to be better if a glow discharge is used.

CONCLUDING REMARKS

The effect of a glow discharge was demonstrated and discussed for obtaining uniform spreading of negative stain in the transmission electron microscopy of cellulose microfibrils. Practical results of negative staining using a glow discharge were already reported by a number of authors.[4,6~9,11,14] Except a few examples,[6,11] the reported irradiation time of specimen grids in a glow discharge is somewhat shorter (less than 30 sec) than the optimal irradiation time in this paper. Because a *Plasmaglo* cleaning system used here has a cathode electrode which is shielded to prevent direct electron bombardment, that is to say, bombarding condition is rather mild and easy to be controlled.

White and Brown, Jr. used Bacitracin as a wetting agent for negative staining with uranyl acetate and obtrained high-resolution images which demonstrate visual characterization of the process of enzymatic hydrolysis of cellulose.[15] In our report here, however, uranyl sulfate was used as a negative stain because of its high solubility in water, high stability for weeks at room temperature and high density.[11] In our preliminary experiments, negative staining using uranyl sulfate combined with Bacitracin was not applicable for the high-resolution electron microscopy of cellulose microfibrils. For use of uranyl sulfate as a negative stain, a technique without detergents should be recommended.

Chanzy and Henrissat treated a carbon-coated grid using ultraviolet for electron microscopic study of the enzymic hydrolysis of *Valonia* cellulose.[16] We also tried such a technique using ultraviolet for uniform spreading of negative stain, but could not obtain good results probably because the power of a ultraviolet source was not enough. Furthermore, it is recommended that irradiation by ultraviolet should be done under vacuum.

The electron irradiation dose necessary for complete disapperance of all crystalline reflections on the diffraction pattern is called the total end-point dose (TEPD). The TEPD of native cellulose crystals was measured as 0.002 ~ 0.003 Coulomb/cm^2 at 120 KV and without staining.[17] As is well known, negative staining of a biological material can increase the TEPD of the material. For example, in the case of a catalase crystal, the TEPD of an unstained and unfixed wet plate crystal is 0.002 Coulomb/cm^2 at 200 KV[18] and that of catalase stained with uranyl acetate is 1.0 Coulomb/cm^2 or more probably at 100 KV.[19] Even if the difference of electron energy is neglected, the effect of negative staining is apparent and marvelous, because a negative staining agent can enter into a unit cell and support the crystal inside. In the case of a cellulose microfibril, the situation is quite different, that is to say, a staining agent can not enter into a crystallite (namely, an elementary fibril or a protofibril if it exists) but supports it only outside. Thus, it is quite interesting whether negative staining can affect the TEPD of cellulose crystals or not. Finally, it is recommended that air bubbles in the aqueous solution of staining agent should be removed with an aspirator just before use, to suppress their effect in the stain (see Figure 1).

REFERENCES

(1) D.W. Gregory and B.J.S. Pirie, "Proc. Fifth Eur. Congr. Electron Microsc., Manchester," p. 234 (1972).
(2) D.W. Gregory and B.J.S. Pirie, *J. Microsc.*, **99**, 261 (1972).
(3) H.E. Huxley and G. Zubay, *J. Mol. Biol.*, **2**, 10 (1960).
(4) E. Namork and B.V. Johansen, *Ultramicrosc.*, **7**, 321 (1982).
(5) J. Trinick and A. Elliott, *J. Microsc.*, **126**, 151 (1982).
(6) S. Fleischer, B. Fleischer and W. Stoeckenins, *J. Cell. Biol.*, **32**, 193 (1967).
(7) K. Fukai, Y. Hosaka and A. Nishimura, *J. Electron Microsc.*, **21**, 331 (1972).
(8) H. Akahori and T. Fukuoka, *J. Electron Microsc.*, **24**, 49 (1975).
(9) J. Dubochet and M. Groom, in "Advances in Optical and Electron Microscopy," vol. 8, ed. R. Barer and V.E. Cosslett, Academic Press, London, p. 107 (1982).
(10) I. Ohad, D. Danon and S. Hestrin, *J. Cell. Biol.*, **12**, 31 (1962).
(11) L.F. Estis, R.H. Haschemeyer and J.S. Wall, *J. Microsc.*, **124**, 313 (1981).
(12) B.V. Johansen, *Micron*, **5**, 209 (1974).
(13) M. Tsuji and R.St.J. Manley, *Colloid and Polymer Sci.*, **262**, 236 (1984).
(14) T. Goto, Ph.D. Thesis, Kyoto University (1976).
(15) A.R. White and R.M. Brown, Jr., *Proc. Natl. Acad. Sci. USA*, **78**, 1047 (1981).
(16) H. Chanzy and B. Henrissat, *Carbohydrate Polymers*, **3**, 161 (1983).
(17) M. Tsuji, S.K. Roy and R.St. J. Manley, *J. Polym. Sci.: Polym. Phys. Ed.*, **23**, 1127 (1985).
(18) V.R. Matricardi, R.C. Moretz and D.F. Parsons, *Science*, **177**, 268 (1972).
(19) R.M. Glaeser and L.W. Hobbs, *J. Microsc.*, **103**, 209 (1975).